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In vitro Regeneration and Optimization of conditions for transformation methods in Pearl millet, *Pennisetum glaucum* (L.)

Jalaja N1*, Maheshwari P2, Naidu K. R1 and Kavi Kishor P. B2

1Department of Biotechnology, Vignan’s University, Vadlamudi- 522213, INDIA.
2Department of Genetics, Osmania University, Hyderabad, India

Abstract

Pearl millet is a dual-purpose crop used for grain and fodder and is grown primarily in Asia and Africa, where it occupies some 27 million ha. It is capable of growing on some of the poorest soils in dry, hot regions of Africa and Asia, where, as a poor man’s source of dietary energy, it sustains a large proportion of the populace. It is also grown in other countries where, under relatively more favorable conditions, it provides grain for bullocks, dairy cows, and poultry. Downy mildew caused by *Sclerospora graminicola* (Sacc.) J. Schroet. is the most widespread and destructive disease of pearl millet causing severe economic losses. New genes can be introduced into this plant through *Agrobacterium* mediated and bombardment genetic transformation for its genetic improvement, which is dependent on the availability of suitable *in vitro* techniques. An efficient regeneration system has been developed for *in vitro* culture of pearl millet (*Pennisetum glaucum* L.) from the immature inflorescence. High frequency callus and shoot regeneration was obtained on Murashige and Skoog nutrient agar medium supplemented with 2mg/l 2,4-D and 0.2 mg/l NAA, 2 mg/l Kinetin and 30 g/l sucrose. On transfer to soil, the regenerated plantlets survived and appeared to be morphologically similar to the normal seed-grown plants. Histological analysis revealed the *de novo* origin of shoots from embryogenic callus in *in vitro* cultured pearl millet. Parameters affecting transformation were optimized by assaying phosphinothricin resistance to transformed calli and basta test for these leaves of plantlets after transferring to pots. These tissues appear to be susceptible to *Agrobacterium* infection and Particle gun flow mediated transformation carrying pCAMBIA2300 with osmotin and chitinase double construct and pPUR with bar genes, as well as shoot multiplication. The embryogenic callus was found competent to take up the DNA, which was monitored by transient bar gene with GV2600 at 0.6 O.D for *Agrobacterium* infection and 1µg/µl plasmid DNA from *E.coli* for co-bombardment was found to be compatible in giving transgenics.

Keywords: co-bombardment transformation, phosphinothricin, basta, regeneration, pearl millet, *Pennisetum glaucum*, immature inflorescence.

Abbreviations

NAA α-naphthaleneacetic acid; BAP 6-Benzylaminopurine; IAA Indole-3-acetic acid; TDZ Thidiazuron; 2,4-D 2,4-dichlorophenoxyacetic acid; NaCl- sodium chloride; PPT- Phosphinothricin

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*Corresponding author: Dr. Jalaja N, Assistant Professor, Department of Biotechnology, Vignan’s University, Vadlamudi- 522213, INDIA, Email: jalajanaravula@gmail.com
Introduction
Pearl millet is a cross pollinated annual C4 crop species that originated in West Africa and introduced into India some 2000 years ago. It is the fifth most important cereal crop and most important millet occupying 55% of global millet production. As the world population continues to increase, food supplies must also grow to meet nutritional requirements. One means of insuring stability of food maintenance is to limit yield loss caused by plant pathogens mainly fungus, bacteria and virus.

Aiming at mass propagation and further quality improvement, micropropagation protocols for this *Pennisetum glaucum* have been tried earlier [1] followed by the reports on regeneration from aseptically grown seedling from immature inflorescence explants [2]. However, there was no convincing report in terms of high frequency multiple shoot regeneration. There are reports of somatic embryos obtained from the immature and mature embryos derived calli of *Pearl millet*, which could not be established into plantlets [3]. It was reported earlier that *in vitro* regeneration in this genus is genotype-dependent and the material is recalcitrant to regeneration [4]. There are reports of regeneration in the other varieties of pearl millet (*Pennisetum glaucum*), Madhavi Latha et al. 2005 [5] reported regeneration in pearl millet. However, the shoot tip callus use of the same regeneration protocol was not effective in regenerating genotypes of 81B-P3 indicating that different cultivars need varied growth regulator combinations. There are no reports on genotype independent regeneration from immature inflorescence of *Pennisetum glaucum*.

Plant intrinsic responses that can be engineered to attain a wider, more durable resistance include the Hypersensitive Response (HR) and systemic acquired resistance (SAR). Although these phenomena are complex, plant genes encoding cell wall degrading enzymes, especially chitinases and osmotin have been used to alter plant resistance to fungal pathogens. But no single gene can give an adequate level of resistance and very few reports exist for resistance to multiple pathogens. It is expected that the transgenic use of chitinase and also osmotin should produce a high level of resistance in crop plants against a variety of fungal pathogens.

Many useful characters can be exploited by interspecific crosses between the wild and cultivated species. Due to strong genetic barriers and the inability to produce a viable hybrid, this method did not appear practical. An alternative to overcome this limitation is to introduce suitable new genes through *Agrobacterium* and attempt using the bombardments method was also done simultaneously. There is no report using the biolistic mediated transformation with immature inflorescence callus of pearl millet and the reports were scanty and inconsistent.

Hence, the present study is undertaken with a cultivar of pearl millet (81B-P6) for preliminary transformation and regeneration studies. The susceptibility of embryogenic callus to various
conditions of Agrobacterium and particle inflow gun mediated transformation under varied conditions was studied. Our observations are reported in this manner.

Materials and methods

Preparation of embryogenic callus
Seeds of pearl millet, 81B-P6 (obtained from ICRISAT, Patancheru, Hyderabad, India) were sown in the field and immature inflorescence was collected after 40 days from the plants. The florescence was surface sterilized with 0.1% HgCl$_2$ and cultured on 2mg/l 2,4-D containing MS Basal medium solidified with 3% sucrose and agar (2%) in tubes as slants. The tubes were inoculated with immature inflorescence and thereafter maintained at 25±2°C with dark condition in a tissue culture room. The callus was subcultured for every 15 days and maintained for atleast 50 days by repeating subculturing on MS Basal medium with 0.1mg/l NaCl to generate the embryogenic calli. This embryogenic calli was used as explants for transformation studies.

Shoot regeneration and rooting
Somatic embryos obtained were transferred on the MS basal containing 0.1mg/l TDZ media for initiation of shoots and maintained on it for 20 days, were later transferred onto regeneration media supplemented with 2mg/l kinetin in combination with 0.2mg/l NAA. The influence of different concentrations of kinetin and BAP was tested for shoot regeneration from this callus. The shoots obtained were rooted in MS medium fortified with 0.2 mg/l IAA.

Studies on calli for antibiotic sensitivity
To test the sensitivity of kanamycin, phosphinothricin and cefotaxime, callus cultures were subcultured onto the petri plates containing MS media with different concentrations of kanamycin (0-300 mg/l) and phosphinothricin (0-20 mg/l) separately. It was found that callus cultures survived even at 300 mg/l kanamycin. This shows that the pearl millet calli are not sensitive to kanamycin. Regarding phosphinothricin, the tissues were subjected to 0-20 mg/l. Seventy per cent of callus tissues died even at 5 mg/l phosphinothricin level, and the tissues turned dark brown at 7 mg/l and died eventually. The frequency (%) of killed embryogenic calli with different concentrations of PPT was recorded. The lethal dose (LD) of PPT at which no calli could survive was determined. Therefore, 5 mg/l phosphinothricin has been taken as a threshold level. Calli were able to tolerate upto 350 mg/l cefotaxime and hence this concentration was used to inhibit the growth of Agrobacterium in the shoot regeneration media.

Cloning of vectors in Agrobacterium
A. tumefaciens strains (GV2600) were transformed by freeze-thaw method [6] using the binary vector pCAMBIA2300 with osmotin and chitinase and pCAMBIA 1300 that contains the plant selection marker bar gene (that confers phosphinothricin resistance), under the control of CaMV
35S promoter. The bar gene, which encodes phosphinothricin acetyl transferase (PAT) which activates by acetylation of the active component of bialaphos, phosphinothricin (PPT) in its T-DNA region. This intron is spliced only during eukaryotic expression. The transformed colonies were selected on solid Luria Agar medium containing 50 mg/l rifampicin and 100 mg/l kanamycin and confirmed the insert with HindIII, PstI, Hind III and Eco-RI digestion.

**Constructs used for genetic transformation**

Plasmids used for the co-bombardment were pCAMBIA2300 double construct rice class I endochitinase gene and osmotin gene from *brassica* and pPUR with bar gene from *Streptomyces hygroscopicus* were isolated with concentration of 1µg/l.

**Agrobacterium mediated transformation protocol**

Liquid cultures were initiated by inoculating a single colony of the bacterial strains harboring this plasmid in YEP medium containing antibiotics. The cultures were grown overnight on a rotary shaker at 28°C at 200 rpm. Bacterial concentrations were adjusted to desired O.D in a spectrophotometer at 600 nm. The bacterial suspensions were centrifuged at 6000 rpm for 10 min at 4°C. The pellet was resuspended in plan MS Basal medium and is used for infection. Simultaneously the embryogenic callus was cultured on liquid MS Basal for 3h and was inoculated to bacterial suspension. The callus was suspended in the bacterial suspension for 15 min, blotted and cultured on co-cultivation media (MS Basal + Acetosyringone). The cultures were co-cultivated in dark for 2-days and washed with cefotaxime and transferred to selection media which contain MS Basal + Cefotaxime + Phosphinothricin. Then after 15 days the callus was transferred to regeneration media (MS Basal + Kinetin 2mg/l + NAA 0.2mg/l) containing phosphinothricin 5mg/l concentration. The parameters like the train of *Agrobacterium* harboring pCAMBIA2300 binary plasmid vector, cell density of bacterial culture (O.D : 0.5), were standardized in transient transformation studies. In some cultures, keeping other parameters constant, the phenolic compound, acetoxyringone was added at a concentration of 100 µM to the bacterial suspensions prior to use and all the cultures were compared to transient transformation efficiency.

**Co-bombardment mediated genetic transformation**

**Plant materials and establishment of embryogenic calli for bombardment**

Embryogenic calli were precultured in MS medium containing 2 mg/l of 2, 4-D, 10mM NaCl, 50mg/l tryptophan and 0.2 M mannitol and 0.2M sorbito (as an osmoticum treatment) for 24 h in the dark before bombardment. Plasmid DNAs of pCAMBIA2300 containing osmotin and chitinase genes and pCAMBIA1300 containing Bar gene was isolated from *E.coli* cultures. Spermidine 100mM and Calcium chloride 2.5M stock was prepared. Gold particles (1.0um diameter) 50mg/l was made ready. 12µ1 of DNA-coated microcarrier suspension was loaded into the center of a macrocarrier and used for bombardment.

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Establishment of parameters for particle bombardment

Optimization of the physical parameters for particle bombardment was carried out under the following conditions, acceleration pressure (900, 1100 and 1350 psi), distance from rupture disk to the macrocarrier (3, 7 and 15 mm), distance from macrocarrier to target tissue (7, 8 and 12 cm), vacuum pressure (24, 26 and 28 in Hg), particle type (gold and tungsten), coating agents (spermidine, CaCl$_2$ and both), number of bombardments (single, double and triple) and time of partial desiccation prior to bombardment (0, 30 and 60 min). Other DNA and biological parameters included were plasmid type (pCAMBIA 2300 and pCAMBIA 1300), DNA concentration (0.5, 2.5, 12.5 and 25 µg per bombardment), tissue type (embryo-genic callus, somatic embryos, shoot tips), osmoticum type (mannitol, sorbitol, sucrose and glucose) and osmoticum concentration (0.2, 0.4, 0.6, 0.8 M mannitol).

Plasmid DNA was precipitated into gold or tungsten particles and bombarded according to the protocols supplied for the Biolistic PDS-1000/He particle delivery system (BioRad, USA). While vigorously vortexing 50 µl of particle solution, 10 µl of DNA, 50 µl of 2.5 M CaCl$_2$ and 25 µl of 0.1 M spermidine and 20µl of dd.H$_2$O.

Culture medium and conditions for plant regeneration

After bombardment with pCAMBIA2300 double construct and pPUR with bar gene immature inflorescence derived embryogenic calli were cultured for 2 weeks on MS Basal medium supplemented with TDZ (0.1mg/l), Kinetin (2mg/l) and Naphthalene acetic acid (NAA 0.2mg/l) with Phosphinothricin and the pH was adjusted to 5.8 before autoclaving. Various combinations and concentrations of growth regulators were used to study regeneration. All the cultures were incubated under standard cultural conditions at 16ºC in light. The number of shoots produced per regenerating explant was recorded at regular intervals.

During the culture, the samples were periodically fixed in acetic acid and alcohol (1:1). Histological sections were taken using a microtome and histological photographs using Nikon Eclipse E 800 Light Microscope (Japan) fitted with Nikon DXN 1200C digital camera.

All the experiments were replicated two times with more than 300 explants in each replication. The significance of the tests was done by Analysis of variance with M-stat.

Rooting and Hardening

Regenerated shoots, about 2-3 cm in length were excised and cultured on MS basal medium for root induction. Rooted shoots were hardened under growth room conditions for 15-days in plastic cups containing a mixture of vermiculite and vermicomposite in the ratio of 1:3 and covered with polythene bags to maintain high humidity. After 25-30 days the acclimatized plants were transferred to lay pots containing soil and were grown to maturity in the green house.
**Molecular Characterization of Transformed Plants**

**Amplification of Chitinase, Osmotin and Bar gene**

PCR reaction was setup by using standard PCR reaction conditions by following the Molecular Cloning Manual by Sambrook and Russel (2001) and the reaction was run in a thermocycler (Perkin Elmer- 2400). PCR amplifications were carried out starting with an initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds for Bar primers and 59°C for 45 seconds for chitinase primers and 58°C for 45 seconds for osmotin primers and 61°C for 45 seconds for Cam35S and PolyA and extension at 72°C for 45 min. Positive controls for bar gene and chitinase were also included. Amplified DNA fragment was separated by gel electrophoresis in 1% agarose gel and images (Biorad Gel documentation) were stored after staining with ethidium bromide.

**RT-PCR of Chitinase and Bar genes**

An amount of 5 µg of total RNA was taken for first strand cDNA synthesis using oligo-dT (20) by using M-MuLV reverse transcriptase (MBI Fermentas, Germany), following manufacturer’s instructions. After the first strand cDNA synthesis, Polymerase chain reaction was performed for the amplification of specific chitinase gene sequence using gene specific primers. Each PCR aliquot was mixed and the PCR reactions were performed in Perkin Elmer machine (Waltham, Massachusetts 02451, USA) or Eppendorf Mastercycler Gradient (Germany) and / or MJ Research Inc., (USA). The standard reaction conditions were carried out as mentioned earlier. An aliquot from the amplified PCR product was used to analyse on 1% agarose gel to check the amplification.

**Southern Blotting Analysis**

For southern hybridization analysis, the genomic DNA (15-20 µg) from each of the putative transformants were separately digested with HindIII and EcoRI that has one restriction site in the pCAMBIA-chitinase and pPUR Bar gene to ascertain the integration pattern based on size separation. The digested DNA was run on 0.8% agarose gel to separate the DNA fragments that were transferred onto nylon membranes (Hybond N+, Amersham Biosciences, UK) using standard protocols [7]. The labeling, hybridization and detection methods were performed according to the manufacturer’s instructions. The gene integration was confirmed by digesting the pCAMBIA2300 with chitinase and osmotin and pPUR Bar plasmids with HindIII restriction enzyme, which served as a positive control. Non-transformed plants (tissue culture raised) were also loaded and they served as negative controls.

**Statistical analyses**

All the data were analyzed for analysis of variance (ANOVA) using M-Stat and the treatment means were compared using sigma plot9.0 software. All experiments were carried in a completely randomized design.
Results and discussion

Callus, shoot regeneration and elongation

Callus initiation was observed in 2mg/l 2, 4-D containing medium. Embryogenic callus growing in the presence of 0.01mg/l TDZ was transferred to MS medium containing kinetin and NAA for plantlet regeneration. A combination of kinetin and NAA gave 97.5% for multiple shoot regeneration. An attempt was made in the present study to initiate callus from the immature embryos, mature embryos and shoot tips of six lines of pearl millet (Table - 1a, 1b). But the calli from the two lines (843B-P2 and 81B-P6) regenerated shoots or plantlets with 3-5% frequency and the number of shoots formed per callus mass was only 3-4 (data not shown). Therefore, immature inflorescences were used as an explants source for callus induction and multiple shoot formation. In the present study, though 2, 4-D induced the embryogenic callus, the frequency was very less. Incorporation of NaCl into the callus proliferating medium followed by culture into thiadiazuron appeared essential for somatic embryogenesis (Figure 1). Callus induced on 2, 4-D was often found to be difficult to maintain in an embryogenic state for long periods and also for shoot differentiation especially in Pennisetum [8].

Multiple shoot regeneration was observed from immature inflorescence callus of culture on the regeneration medium. Shoot buds appeared after two weeks and shoots were visible by 15 days of culture on the medium. A combination of 0.2 mg/l NAA and 0.5 mg/l Kinetin with 30 g/l sucrose promoted 80% shoot regeneration frequency with the immature inflorescence callus (Table - 2a, 2b). Upon sequential subculture containing medium, the number of shoot buds could be increased to an average of 10 per explant.

In media supplemented with NAA (0.1, 0.3, 0.5 mg/ l) and kinetin (1.0, 2.0, 4.0 mg/ l), regeneration was developed with the formation of green nodular structures after 4 weeks from the callus. Kinetin was more suitable for regeneration. The regeneration on NAA and BAP was also observed but the percentage of regeneration was less. Data on shoot tip development were collected at regular intervals. The callus was become brown and smooth in further subculture of callus.

Shoots were excised and transferred to MS basal medium with 0.2mg/l IAA for root differentiation. They developed roots with 70% frequency. Well-developed plantlets were transferred to pots containing sand and soil mixture in a ratio of 1:3. Plants were covered with glass beakers to maintain humidity and watered with Hoagland nutrient solution at 3-4 days intervals. Beakers were removed after two weeks of transfer to the pots. The frequency of survival was 60% and morphologically the plants dwarf that of seed-raised plants.
Table 1a: Percent frequency of callus initiation from immature inflorescences of different genotypes of Pennisetum glaucum (L) on MS medium supplemented with auxins

<table>
<thead>
<tr>
<th>Growth regulators(mg/l)</th>
<th>Genotypes</th>
<th>81B-P6</th>
<th>843B-P2</th>
<th>863B-P3</th>
<th>P1449</th>
<th>PT732</th>
<th>TIFT-D238</th>
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</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>68.3 (±1.4)</td>
<td>77 (±1.4)</td>
<td>27.7 (±1.5)</td>
<td>8.0 (±1.0)</td>
<td>28.6 (±0.3)</td>
<td>10.0 (±1.0)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>95.3 (±2.9)*</td>
<td>94 (±3.0)*</td>
<td>71.0 (±2.1)</td>
<td>13.6 (±3.4)</td>
<td>36.66 (±3.3)</td>
<td>19.3 (±0.6)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>26.3 (±2.4)</td>
<td>16.7 (±2.4)</td>
<td>7.0 (±2.0)</td>
<td>3 (±1.5)</td>
<td>26.33 (±1.4)</td>
<td>13.3 (±0.3)</td>
</tr>
<tr>
<td>Dicamba</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>26 (±3.0)</td>
<td>36.0 (±3.0)</td>
<td>7.0 (±0.6)</td>
<td>6.3 (±0.8)</td>
<td>16.7 (±2.0)</td>
<td>2.7 (±1.2)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>65 (±2.8)</td>
<td>67.0 (±2.0)</td>
<td>26.0 (±3.0)</td>
<td>10.0 (±2.8)</td>
<td>22.3 (±1.4)</td>
<td>7.0 (±2.1)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>7.6 (±1.4)</td>
<td>5.6 (±2.3)</td>
<td>-</td>
<td>5.0 (±2.5)</td>
<td>11.7 (±1.6)</td>
<td>3.0 (±1.7)</td>
</tr>
<tr>
<td>Picloram</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>8.7 (±1.3)</td>
<td>8.7 (±0.7)</td>
<td>3 (±1.5)</td>
<td>5.6 (±2.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>21 (±2.0)</td>
<td>27.6 (±1.5)</td>
<td>18 (±1.0)</td>
<td>10.6 (±0.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>9.7 (±1.5)</td>
<td>7.7 (±1.2)</td>
<td>6.7 (±0.7)</td>
<td>2.7 (±1.7)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Analysis of variance was performed using M-Stat software.
Values are significant among different genotypes and growth regulators.
Values are significant at P ≤ 0.6.

Table 1b. Analysis of Variance showing significance between genotype, rooting media and regeneration media for callus initiation from immature inflorescences of different genotypes of Pennisetum glaucum (L) on MS medium supplemented with auxins

<table>
<thead>
<tr>
<th>K value</th>
<th>source</th>
<th>Degree of Freedom</th>
<th>Sum of squares</th>
<th>Mean value</th>
<th>F value</th>
<th>Prob</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Replication</td>
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<td>6.021</td>
<td>1.6996</td>
<td>0.2052</td>
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<tr>
<td>2</td>
<td>Factor A</td>
<td>1</td>
<td>93.521</td>
<td>93.521</td>
<td>26.3991</td>
<td>0.0000</td>
</tr>
<tr>
<td>4</td>
<td>Factor B</td>
<td>1</td>
<td>1598.521</td>
<td>1598.521</td>
<td>451.2317</td>
<td>0.0000</td>
</tr>
<tr>
<td>6</td>
<td>AB</td>
<td>1</td>
<td>744.187</td>
<td>744.187</td>
<td>210.0698</td>
<td>0.0000</td>
</tr>
<tr>
<td>8</td>
<td>Factor C</td>
<td>5</td>
<td>3224.354</td>
<td>644.871</td>
<td>182.0346</td>
<td>0.0000</td>
</tr>
<tr>
<td>10</td>
<td>AC</td>
<td>5</td>
<td>4406.354</td>
<td>881.271</td>
<td>248.7658</td>
<td>0.0000</td>
</tr>
<tr>
<td>12</td>
<td>BC</td>
<td>5</td>
<td>475.354</td>
<td>95.071</td>
<td>26.8367</td>
<td>0.0000</td>
</tr>
<tr>
<td>14</td>
<td>ABC</td>
<td>5</td>
<td>870.688</td>
<td>174.138</td>
<td>49.1557</td>
<td>0.0000</td>
</tr>
<tr>
<td>-15</td>
<td>Error</td>
<td>23</td>
<td>81.479</td>
<td>3.543</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>47</td>
<td>11500.479</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factor A – Genotypes; Factor B – Rooting media; Factor C – Regeneration media

Effect of age of inflorescence
The age of the immature inflorescence and regeneration capacity showed an inverse relationship with the percentage of cultures showing shoot regeneration. In all the experiments aimed at testing the effect of the age of explants, the number of explants producing callus declined with the increase in age of the growth of the plant acting as donors for the immature inflorescence explants (Figure 1). As far as the plant age is concerned, explants from 25- day old seedlings showed the highest response. Also, we did not notice significant variation between 25 and 35-day-old plants. After 35 days, there was a significant decline in callus differentiation with
increased age of plant acting as explant donors. However, callus produced on explants from 25-day-old plants was higher embryogenic compared to the callus prepared from 40-day-old seedlings. These observations clearly suggest that the 5-day-old seedlings are the best source for generating the embryogenic callus in \textit{P. glaucum} from immature inflorescence.

Table 2a: Frequency of shoot differentiation from embryogenic callus of different pearl millet genotypes (843B-P2 and 81B-P6) and the number of shoots formed per callus mass

<table>
<thead>
<tr>
<th>Plant growth regulators (mg/l)</th>
<th>% Frequency of shoot differentiation</th>
<th>No. of shoots/callus mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotypes</td>
<td>843B-P2</td>
</tr>
<tr>
<td>BAP</td>
<td>IAA</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Kinetin</td>
<td>IAA</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
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<td></td>
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<td>Kinetin</td>
<td>NAA</td>
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<td></td>
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</tr>
<tr>
<td>BAP</td>
<td>NAA</td>
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<tr>
<td></td>
<td></td>
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</tbody>
</table>

*Analysis of variance was performed using M-Stat software. Values are significant among different genotypes and growth regulators for shoot differentiation. Values are significant at P ≤ 0.06 for % Frequency of shoot differentiation, Values are significant at P ≤ 0.05 for No. of shoots/callus mass.

Table: 2b. Analysis of Variance showing significance between Genotype, Growth regulator and concentration of shoot differentiation from embryogenic callus of different pearl millet genotypes (843B-P2 and 81B-P6) and the number of shoots formed per callus mass

<table>
<thead>
<tr>
<th>K value</th>
<th>Replication</th>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Sum of Squares</th>
<th>F Value</th>
<th>prob</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Replication</td>
<td>1</td>
<td>16.6460</td>
<td>16.6460</td>
<td>4.3388</td>
<td>0.0421</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Factor A</td>
<td>5</td>
<td>9103.3570</td>
<td>1820.6710</td>
<td>474.5667</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Factor B</td>
<td>2</td>
<td>27178.0480</td>
<td>13589.0240</td>
<td>3542.0438</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AB</td>
<td>10</td>
<td>5670.5040</td>
<td>567.0500</td>
<td>147.8044</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Factor C</td>
<td>2</td>
<td>8862.6540</td>
<td>4431.3270</td>
<td>1155.0464</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AC</td>
<td>10</td>
<td>2993.3010</td>
<td>299.3300</td>
<td>78.0218</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>BC</td>
<td>4</td>
<td>4833.7510</td>
<td>1208.4380</td>
<td>314.9851</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>ABC</td>
<td>20</td>
<td>2136.4550</td>
<td>106.8230</td>
<td>27.8439</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>-15</td>
<td>Error</td>
<td>53</td>
<td>203.3340</td>
<td>3.8360</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>107</td>
<td>60998.0490</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factor A – Genotype; Factor B – Growth regulator; Factor B – Concentration
Figure: 1 Different stages of *in vitro* plant regeneration from immature inflorescence derived callus cultures of the pearl millet genotype 81B-P6.

(a) Embryogenic callus from immature inflorescence; (b) Shoot initiation; (c) Proliferation of shoots (d, e, f) Multiple shoots from each calli

Figure: 2 Histological studies on regeneration from immature inflorescence and shoot-tip derived calli of *Pearl millet*

(a) Globular shaped somatic embryos (25X); (b and c) Shoot differentiation surrounded by leaf primordia and leaf primordia (80 X); (d) Further divisions leading to a callus initiation (100X); (e) Multiple shoot apices (100 X); (f) Regenerated shoots from the abaxial side of the explant as seen under Microscope
Histological analysis

Histology of the shoot forming calli revealed multiple shoot tips surrounded by leaf primordial (Figure 2). Cross sections of the explants that were fixed at different stages revealed that the regenerated buds developed from the epidermal cells and these cells divided initially followed by the divisions in the inner rows of cells and the stimulated cells beneath developed shoots. The developed shoot initiation could be clearly observed after 20th day of inoculation to regeneration media. Well formed shoots could be observed by 40th day of the culture.

Transformation of callus with pCAMBIA2300 with osmotin and chitinase double construct and pCAMBIA1300 with bar gene construct

Optimal conditions for transformation based on transients by phosphinothricin selection that represents early infection were identified using Agrobacterium strain harboring binary vector pCAMBIA. In the preliminary examinations, the ability of the strains EHA105 and LBA4404 to transfer genes was compared by looking for transient GUS activity.

Phosphinothricin could be attributed to the actual expression of the bar gene, since it requires the removal of catalase intron, which interrupts the gusA sequence during RNA processing by the eukaryotic cells [9]. Multiple factors play a role in efficient T-DNA transfer and transformation of callus explants. The influence of Agrobacterium strain, bacterial concentration, acetosyringone and co-cultivation time and cefotaxime concentration and washes were examined (Table - 3).

Addition of acetosyringone

Acetosyringone (AS) is known to enhance the transfer of T-DNA from Agrobacterium to plant cells in many plants like cotton etc. The primary step in genetic transformation is the attachment of Agrobacterium to the host plant. This is facilitated by the genes present on the bacterial chromosome. Compounds like AS are known to induce Agrobacterium virulence genes that help in transfer of T-DNA to the host plant genome at the site of injury. In many cases, AS is known to induce expression of vir genes, which is necessary for the generation of T strands and their transfer to the plant cells. We observed that the addition of 100µM AS increased the blue color intensity at the petioles. This indicates that the vir- inducing compounds such as AS had a positive effect on the T-DNA transfer in white jute.

Table: 3 - Transformation of pearl millet using Agrobacterium strain GV2260 containing pCAMBIA 2300 with osmotin and chitinase double construct and pCAMBIA 1300 with bar gene constructs

<table>
<thead>
<tr>
<th>Number of embryogenic callus Infected</th>
<th>Co-cultivation period (day)</th>
<th>Number of explants survived on selection media after first week</th>
<th>Number of explants survived on selection media after second week</th>
<th>% frequency of survival of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>2</td>
<td>30</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>
Table: 4 Efficiency of biolistic transformation of Pearl millet

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Transgenics</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of embryogenic calli bombarded</td>
<td>100</td>
</tr>
<tr>
<td>No. of resistance calli</td>
<td>70</td>
</tr>
<tr>
<td>No. of regeneration plants</td>
<td>60-65</td>
</tr>
<tr>
<td>No. of shoots per plant</td>
<td>10-20</td>
</tr>
<tr>
<td>No. of plantlets survived on rooting</td>
<td>50</td>
</tr>
<tr>
<td>No. of plants survived after transplantation</td>
<td>40</td>
</tr>
<tr>
<td>No. of plants maintained in net house</td>
<td>10</td>
</tr>
<tr>
<td>Transformation efficiency (%)</td>
<td>50</td>
</tr>
</tbody>
</table>

Co-bombardment mediated transformation with pCAMBIA osmotin and chitinase and pPUR bar gene

Particle-inflow-gun mediated genetic transformation, optimum parameters were standardized for osmotin and chitinase double construct mediated genetic transformation. The various established parameters, viz., 18 kg/cm² of helium pressure; 7 cm of target distance; 2 μg of DNA/mg tungsten particles; 0.25 M sorbitol and 0.25 M mannitol as osmoticum; 8 h of pre-bombardment osmotic treatment; and 24 h of post-bombardment osmotic treatment. However, under identical conditions, about 60% transformation frequency was observed using 10 kg/cm² of helium pressure; 12 cm of target distance; 2 μg of DNA/mg tungsten particles; 0.2M sorbitol and 0.2M mannitol as osmoticum; 6 h of pre-bombardment osmotic treatment; and 24 h of post-bombardment osmotic treatments with 0.2 M each of sorbitol and mannitol. Selection and regeneration of transformed calli Phosphinothricin (PPT) sensitivity test on embryogenic calli specified 5 and 10 mg/l, respectively, as LDS50 and LD100 doses. The embryogenic calli co-bombarded with chitinase and osmotin 35S and pBar 35S constructs, were cultured on MS medium supplemented with 2,4-D + PPT. The calli exhibiting PPT-resistant sectors were separated and regenerated (Figure 3) on MS medium supplemented with BAP + IAA. Out of 30 selected calli, only 12 resistant calli could give rise to plant regeneration (Table - 4). The regenerated putative transformants with well-developed roots on MS Basal medium were established in pots (Figure 4) and grown to maturity in the net house.
The plant expression vector pPUR, gene, has been used for optimising the bombardment parameters for transformation of embryogenic calli. Maximum frequency (70%) was produced using 18 kg/cm$^2$ helium pressure; at 12 cm target distance; 2 μg DNA/mg tungsten particles; 8 h pre- and 24 h postbombardment osmotic treatments with 0.2M sorbitol and 0.2M mannitol owing to the cumulative effects of optimized parameters. However, <18 kg/cm$^2$ helium pressure, >12 cm target distance and 2 μg DNA/mg tungsten particles produced lesser frequency of phosphinothricin resistant calli, probably, because of less efficient dispersion of particles into the target tissue. In sorghum, a higher helium pressure and lower target distance was found to cause increased tissue damage leading to low frequency of transformation. However, >1 μg DNA/mg tungsten particles invariably decreased the frequency of gene expression due to particle agglomeration, which accorded with the results recorded for maize by Klein et al. [10].

**Basta test for bar gene transformation**

Expression of bar gene in putative (T$_0$) transformants Four of the putative transformants subjected to Basta (0.25%) leaf dip assay disclosed tolerance to the herbicide. The leaves of three Basta-tolerant transformants retained their healthy green appearance, whereas leaves of susceptible untransformed control plants were scorched within 72 h of Basta treatment.
The high frequency of resistant calli to PPT was observed in the present study, by applying various pre- and post-bombardment osmotic treatments, may be ascribed to the decreased cell damage caused by increased permeability and rapid cell recovery. In earlier reports, similar osmotic treatments were found to increase the efficiency of both transient gene expression and stable genetic transformation in finger millet [5], pearl millet [11], maize [12], wheat [13] and tobacco cells [14].

**Molecular analysis for transformation studies**

Molecular analyses of putative (T₀) transformants PCR was carried out on the genomic DNA isolated from the Basta-tolerant putative transformants and the untransformed control, using primers for bar coding sequence. The various transformants and the positive control (pBar 35S) invariably showed an amplification product of 560 bp. And the PCR for the genes chitinase and osmotin was also with specific primers showed amplification product of 600bp and 750bp. Conversely, no such band was observed in the untransformed control under identical conditions (Figure 5).

**Figure: 5 Amplification of chitinase and bar genes in putative transgenics generated by bombardment**

Utilizing the various optimised parameters for transient expression of the reporter expression gene, stable transgenic pearl millet plants were successfully produced by employing picambia Osmotic and Chitinase 35S and pBar 35S constructs. The four-step selection strategy using PPT was found effective for selecting the transformed calli and for producing stable transgenic plants. These findings are in agreement with those of Goldman et al. (2003) who could minimize the chances of escapes by applying higher concentrations of PPT to different transformed tissues of pearl millet. PCR analysis using bar, chitinase and osmotin primers revealed an amplification of 560bp, 600bp and 700bp fragment (Figure 5) in all the three Basta-tolerant putative transformants, clearly indicating the presence of bar gene in various transformants.

Southern blot analysis was carried out using chitinase coding sequence as a probe on transformants tolerant to Basta and PCR-positive for bar and chitinase and osmotin genes. Three
transformants, showed more than one hybridizable band, while T₀ showed only one hybridizable band. In these transformants, however, the hybridizable bands were of different sizes >1.5 kb. Whereas, no such band was observed in the untransformed control plants (Figure 6).

**Figure: 6 Southern blotting of chitinase gene**

![Southern blotting of chitinase gene](image)

C- Control; NC - Negative Control; 1,2,3 - transformed plants

Total RNA isolated from the leaves of Southern-positive transgenics plants and untransformed control plants was subjected to RT-PCR. A clear band of 600 bp was observed in three transgenics plants. Conversely, no such band was observed in the untransformed control plants.

These four transformants, tolerant to Basta and PCR-positive for bar, were also found Southern positive for the chitinase gene (Figure 7), thereby suggesting the stable integration of pin gene into their genomes. HindIII digested genomic DNA with the pin probe disclosed the presence of hybridizable bands of different sizes >4 kb. Three of the primary transformants, exhibited more than one hybridizable band, while 2T₀ revealed a single hybridizable band, implying that the chitinase gene is integrated at different sites in their genomes. In four transformants, the number of integrated transgene copies were varied as indicated by the variable size and the number of hybridizable bands.

**Figure: 7 Extraction of total RNA and RT-PCR analysis**

(a) RNA isolated from transgenics

(b) RT-PCR of chitinase gene

![Extraction of total RNA and RT-PCR analysis](image)

Legends: Lane - 1- Ladder; Lane - 2 to 7 - Transformed plants
The RT-PCR study was also done for the confirmation of transgenics. The phenomenon of multiple copy integration is commonly encountered in particle-bombardment-mediated genetic transformation of plants [15-18]. In earlier studies, a variable number of incorporated transgene copies ranging between 1 and 20 were reported in diverse crop plants [19-30].

Transient gene expression
The highest infection frequency of immature inflorescence callus was obtained with bacterial strains GV2260 (OD 0.3) and by co-bombarding the callus for particle gun mediated transformation. This resulted in an increase in resistance of the callus to phosphinothricin indicating enhanced gene transfer. These results establish that A. tumifaciens [31] and bombardment can be employed for stable genetic transformation in immature inflorescence callus of pearl millet [32], since plant regeneration from this species is successful.

These experiments are repeated for the development of stable transgenic pearl millet using Agrobacterium and bombardment methods.

Conclusion
Transgenic pearl millet lines expressing osmotin and chitinase gene exhibiting high resistance to downy mildew pathogen, Sclerospora graminicola were produced using Agrobacterium infection and co-bombardment method. Immature inflorescence derived embryogenic calli were co-bombarded with plasmids containing osmotin and chitinase in pCAMBIA2300 and bar gene in pPUR vector driven by CaMV 35S promoter. Bombarded calli were cultured on MS medium with phosphinothricin as a selection agent. Primary transformants showed the presence of both bar and chitinase and osmotin coding sequences as evidenced by PCR and Southern blot analysis, RT-PCR respectively.

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Conflict of Interest
We declare that we have no conflict of interest
References


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